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Suzuki, K. W.; Ueda, H.; Nakayama, K.; Tanaka, M.

CITATION:

Suzuki, K. W. ...[et al]. Spatiotemporal dynamics of stable carbon isotope ratios in two sympatric oligohaline copepods in relation to the estuarine turbidity maximum (Chikugo River, Japan): implications for food sources. Journal of Plankton Research 2013, 36(2): 461-474

ISSUE DATE:

2013-09-26

URL:

http://hdl.handle.net/2433/199858

Spatiotemporal dynamics of stable carbon isotope ratios in two sympatric oligohaline copepods in relation to the estuarine turbidity maximum (Chikugo River, Japan): implications for food sources

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KEYWORDS: copepod; estuarine turbidity maximum; *Pseudodiaptomus inopinus*; salinity; *Sinocalanus sinensis*

20 **ABSTRACT**

21 To improve our understanding of high copepod productivity in the estuarine turbidity maximum
22 (ETM) of the macrotidal Chikugo River estuary in southwestern Japan, we determined stable
23 carbon isotope ratios ($\delta^{13}C$) in the sympatric oligohaline copepods *Sinocalanus sinensis* and
24 *Pseudodiaptomus inopinus* from 2005 to 2006. Terrestrial-plant and phytoplankton detritus always
25 accumulated in the ETM (salinity 0.1–3), whereas outside the ETM phytoplankton dominated
26 especially in the warm season (> 20ºC). In contrast with the year-round concentration of *S. sinensis*
27 in the ETM, *P. inopinus* occurred widely along the upper estuary under phytoplankton-dominated
28 conditions. Terrestrial-plant detritus was characterized by relatively constant $\delta^{13}C$ (ca. -24‰),
29 suggesting that significant spatiotemporal variability in copepod $\delta^{13}C$ was attributable to the feeding
30 of copepods on phytoplankton and/or its detritus. Both copepods held relatively depleted $\delta^{13}C$
31 values in the ETM, reflecting $\delta^{13}C$ in freshwater/oligohaline phytoplankton (< -24‰). However,
32 relatively enriched $\delta^{13}C$ values (> -24‰) associated with meso/polyhaline phytoplankton
33 downstream from the ETM were found only in *P. inopinus*. Although the contribution of
34 terrestrial-plant detritus to copepod production remains to be determined, our results indicate that
35 both copepods selectively utilize freshwater/oligohaline phytoplankton and/or its detritus in the
36 ETM whereas only *P. inopinus* utilizes meso/polyhaline phytoplankton downstream from the ETM.
37

INTRODUCTION

The estuarine turbidity maximum (ETM) develops at low salinities in macrotidal estuaries through the hydrodynamic function of tidal pumping and estuarine circulation (Allen *et al.*, 1980; Uncles *et al.*, 2002). High densities of zooplankton are often associated with high concentrations of suspended solids in the ETM (Castel and Veiga, 1990; Laprise and Dodson, 1994; North and Houde, 2003). The ETM generally serves as a fish nursery, providing better feeding conditions to larval and juvenile fish than other habitats (Dauvin and Dodson, 1990; Sirois and Dodson, 2000; Martino and Houde, 2010). However, phytoplankton production is inhibited in the ETM, as light availability for photosynthesis is severely reduced by the high turbidities that exist here (Irigoien and Castel, 1997; Yokoyama *et al.*, 2012). Therefore, detrital food sources of allochthonous origin (e.g. freshwater and marine phytoplankton and terrestrial plants) have been considered to subsidize high zooplankton productivity in the ETM (Heinle and Flemer, 1975; Heinle *et al.*, 1977; Roman, 1984; David *et al.*, 2006).

In Japan, the oligohaline copepod *Sinocalanus sinensis* occurs only in macrotidal estuaries in the innermost part of the Ariake Sea (Hiromi and Ueda, 1987; Ohtsuka *et al.*, 1995; Ueda, 2005). On the contrary, the copepod *Pseudodiaptomus inopinus* is widely distributed in brackish waters of East Asia (Ohtsuka *et al.*, 1995; Sakaguchi *et al.*, 2011). Although both copepods coexist in the macrotidal Chikugo River estuary, the largest estuary flowing into the Ariake Sea, *S. sinensis* numerically dominates in and close to the ETM throughout the year except in the warm season when *P. inopinus* outnumbers *S. sinensis* (Suzuki *et al.*, 2013). Given the vulnerability of eggs and nauplii to washout from the estuary, large floods characteristic of the warm season are more detrimental to the free-spawning species *S. sinensis* than to the egg-carrying species *P. inopinus* (Suzuki *et al.*, 2012a). Besides their different reproduction strategies, differential food sources could also explain the seasonal alternation of dominance between the two copepods. Terrestrial-plant and phytoplankton detritus continuously accumulates in the ETM and therefore could ensure minimum food for the two copepods throughout the year (Suzuki *et al.*, 2012b). In contrast, phytoplankton,

which proliferates outside the ETM in the warm season, could serve as nutritious food of limited availability (Suzuki *et al.*, 2012b).

To test the hypothesis that the sympatric oligohaline copepods *S. sinensis* and *P. inopinus* depend on differential food sources, we determined stable carbon isotope ratios ($\delta^{13}C$) in the two copepods along the Chikugo River estuary monthly between 2005 and 2006. Photosynthetic pigment concentrations and $\delta^{13}C$ in particulate organic carbon (POC) were also measured in ambient water. Since $\delta^{13}C$ in animals reflect $\delta^{13}C$ in their diet (DeNiro and Epstein, 1978; Fry and Sherr, 1984), spatiotemporal variability in copepod $\delta^{13}C$ was compared with that in POC $\delta^{13}C$. Significant spatiotemporal variability in copepod $\delta^{13}C$ was attributed to the feeding of copepods on phytoplankton and/or its detritus, as phytoplankton is characterized by spatiotemporally variable $\delta^{13}C$ in contrast with relatively constant $\delta^{13}C$ in terrestrial plants (cf. Suzuki *et al.*, 2012b). Differences in $\delta^{13}C$ between copepods and POC were used to evaluate selective feeding and/or assimilation by copepods (cf. Del Giorgio and France, 1996). Dependence on differential food sources is discussed between *S. sinensis* and *P. inopinus* in light of their respective patterns of spatial occurrence relative to the ETM.

METHOD

Study area

The Chikugo River estuary is the largest estuary in the Ariake Sea in terms of both catchment area (2860 km^2) and freshwater discharge (annual median of daily averages: 60 m^3 s^{-1}). The estuarine environment is characterized by one of the largest tidal ranges in Japan (up to 5 m during spring tides). The tidal reach is 4–8 m in depth and 300–1000 m in width at spring high tide, extending to the Chikugo Weir 23 km upstream from the river mouth. Strong tidal currents completely mix the water column during spring tides, whereas partial stratification occurs during neap tides (Suzuki *et al.*, 2007). The ETM is usually located 10–20 km upstream from the river mouth at spring high tide, although it is transported back and forth over a 20-km range along the estuary with the semidiurnal

tidal cycle between high and low tides. Terrestrial-plant and phytoplankton detritus accumulates into the ETM throughout the year, whereas phytoplankton occurs abundantly outside the ETM during the warm season (Suzuki *et al.*, 2012b). Benthic microalgae are considered to be negligible compared with phytoplankton and terrestrial plants (Suzuki *et al.*, 2012b). Large floods occasionally affect the estuary and wash away the ETM especially in the warm season (Suzuki *et al.*, 2009). The spatiotemporal dynamics of the horizontal distribution of *S. sinensis* and *P. inopinus* along the estuary is published elsewhere (Suzuki *et al.*, 2012a). Moreover, the spatiotemporal dynamics of both concentration and origin of POC has been studied in detail relative to the fortnightly tidal cycle (Suzuki *et al.*, 2007), freshwater discharge levels (Suzuki *et al.*, 2009) and the seasonal succession (Suzuki *et al.*, 2012b).

Seven regular sampling stations (R1–R7; Fig. 1) were set up at intervals of 1.5–5.5 km along the lower reaches of the Chikugo River, located between the river mouth and the upper limit of the tidal reach (23 km upstream). Three regular sampling stations (E1–E3; Fig. 1) were set up along the main tidal channel of the river so that E1 was near the river mouth and E3 was at the edge of the tidal flat (9 km offshore). The freshwater discharge was continuously monitored 26 km upstream and the data were uploaded to the web site by the Chikugogawa River Office.

Field sampling

Monthly sampling was conducted at the ten regular sampling stations during the period March 2005 to December 2006. All sampling dates coincided approximately with spring tides. Sampling involved vertical hauls of a plankton net (45-cm mouth diameter, 100-cm long, 0.1-mm mesh aperture) from close to the bottom to the surface at ~50 cm s^{-1}. Zooplankton was preserved in 5% formalin seawater solution for taxonomic analysis of copepods. The volume of water filtered was estimated using a flow meter (2030R, General Oceanics, USA) attached to the mouth of the plankton net. For $\delta^{13}C$ analysis of copepods, zooplankton was immediately frozen on dry ice. Frozen samples were transported to the laboratory and stored at -30ºC until further analysis.

Temperature, salinity and turbidity were also measured from the bottom to the surface at depth intervals of 1 or 2 m using environmental monitoring systems (6920 Sonde and 650 MDS Display, YSI, USA; Compact-CTD, Alec Electronics, Japan). Turbidity was not measured in April and July 2005 and February 2006, due to mechanical faults of the turbidity sensor. Water samples were taken from the surface with a bucket and prefiltered through a nylon screen (0.1-mm mesh aperture) to remove zooplankton and plant debris before being packed in clean bottles. Plankton-net hauls and environmental surveys were started at the uppermost station (R7) and finished at the lowermost station (E3) within 4–5 h around high tide in the morning. To assess temporal changes in copepod $\delta^{13}C$ in response to short-term environmental changes, a series of intensive sampling was conducted once every 5 or 6 days from 8 June to 9 August 2005. This period of the year is considered to represent the warm and wet season in southwestern Japan (Suzuki *et al.*, 2012b). Three extra sampling stations (R2.5, R3.5 and R6.5; Fig. 1) were set to focus on the ETM and its surrounding waters, whereas sampling was not conducted at stations far downstream from the ETM. Given relatively weak mixing in the water column in summer, water samples were taken not only from the surface but also from approximately 1 m above the bottom using a Van Dorn water sampler. The other procedures for collecting biological and physical data were the same for those of the monthly sampling.

Laboratory analysis

Water samples were filtered through Whatman GF/F filters that had been combusted at 400ºC for 3h. Depending on turbidity, water volume for filtration was adjusted (20–1000 ml). Duplicates were made for each water sample to analyze photosynthetic pigments and $\delta^{13}C$ separately, although both analyses were done once for each sample. Filter samples were kept frozen at -30ºC until further analysis. Half of the filters were extracted in the dark for 12 h with 90% acetone. Extracts were measured for chlorophyll *a* and phaeopigment concentrations by the fluorometric method using a calibrated Turner Designs TD 700 fluorometer (Japan Meteorological Agency, 1970). The

fluorescence of phaeopigments, an indicator of plant detritus, was quantified after acidification with HCl. For $\delta^{13}C$ analysis of POC, the other half of the filters were dried at 60ºC for 24 h and acidified by fuming with HCl for 24 h to remove $CaCO_3$. To neutralize the acid, samples were placed in a desiccator with NaOH for more than 72 h and then redried. The processed samples were wrapped separately in tin foil and kept dry in another desiccator.

To estimate density (ind. L^{-1}), adults and copepodids of *S. sinensis* and *P. inopinus* were extracted from the formalin samples and identified under a stereo microscope (× 20–50 magnification). For $\delta^{13}C$ analysis of copepods, the frozen zooplankton samples were thawed slowly and identified to species under the stereo microscope during the period of thawing. Adults and copepodids of *S. sinensis* and *P. inopinus* were pooled by species (~50 individuals) at each station on each sampling date to obtain sufficient material for $\delta^{13}C$ analysis. Pooled samples were rinsed with distilled water and wrapped separately in tin foil before being dried at 60ºC for 24 h. Neither acidification nor lipid extraction was conducted in zooplankton samples. All $\delta^{13}C$ values were determined using a stable isotope ratio mass spectrometer (Delta S, Finnigan MAT, Germany) in the continuous flow mode, equipped with an elemental analyzer (EA1108, Fisons Instrument, Italy). Stable carbon isotope ratios are described as a per mil (‰) deviation from the international standard (Peedee Belemnite) using the following equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X and R represent ^{13}C and $^{13}C/^{12}C$ ratio, respectively. To verify the accuracy of the analysis, $_{DL}$-alanine was used as a secondary standard. Standard deviations for the secondary standard were usually less than 0.1‰ for $\delta^{13}C$. Organic carbon and nitrogen concentrations in each sample were determined simultaneously with $\delta^{13}C$ analysis using the elemental analyzer. The results were used to calculate POC concentrations and organic carbon to nitrogen atomic ratios (hereafter, C:N ratios).

The C:N ratios in copepods were used as an indicator of lipid content, since depleted $\delta^{13}C$ values are frequently associated with a high lipid content (Matthews and Mazumder, 2005; Smyntek *et al.*, 2007). In contrast, enriched $\delta^{13}C$ values are sometimes attributable to a high carbonate content and therefore acidification is recommended by some researchers (Jacob *et al.*, 2005;

Carabel *et al.*, 2006). However, the effect of carbonate on copepod $\delta^{13}C$ was not considered in the present study, because carbonate content was considered to be minor in copepods and acidification might increase inter-individual variation in $\delta^{13}C$ (cf. Bunn *et al.*, 1995). Although each sampling station was represented by a single pair of POC and copepod samples per sampling date, this laborsaving procedure is considered to be accurate enough to survey spatial variation in POC and copepods along the estuary (cf. Suzuki *et al.*, 2012b).

Data analysis

Temperature, salinity and turbidity were averaged through the water column. Average temperature was used to divide a year into cold (< 20ºC) and warm (> 20ºC) seasons. Average salinity was used as an indicator of distinctive water masses, because the distribution of water masses changes considerably along the estuary in response to the fortnightly tidal cycle as well as freshwater discharge levels (Suzuki *et al.*, 2007, 2009). Based on the result of the present study, four water masses were distinguished empirically by salinity ranges of < 0.1, 0.1–3, 3–20, > 20 (without statistics). To assess the spatiotemporal variability in environmental conditions, effects of salinity and temperature on environmental parameters (e.g. turbidity, POC and chlorophyll *a*) were analyzed by two-way repeated-measures analysis of variance (hereafter, two-way ANOVA) on the software JMP Ver. 5 (SAS Institute Inc., USA). In the intensive sampling campaign, surface and bottom values were averaged to determine concentrations of chlorophyll *a*, phaeopigments and POC, and $\delta^{13}C$ in POC. Although all environmental parameters (absolute values for $\delta^{13}C$) were logarithmically transformed to assure preconditions for two-way ANOVA, normality and/or homogeneity of variance were not always satisfied even after the logarithmic transformation. It follows that provisional results might be included in the two-way ANOVA tests.

To evaluate selective feeding and/or assimilation by copepods (cf. Del Giorgio and France, 1996), differences in $\delta^{13}C$ between copepods and POC were calculated by subtracting POC $\delta^{13}C$ from copepod $\delta^{13}C$ at each station on each sampling date (hereafter, $\Delta\delta^{13}C$). The significance of

correlations of copepod parameters ($\delta^{13}C$, C:N ratios and $\Delta\delta^{13}C$) with environmental parameters (e.g. salinity, turbidity and POC) was tested by Spearman's correlation coefficient. Kruskal-Wallis test was used to compare $\Delta\delta^{13}C$ values among salinity ranges (< 0.1, 0.1–3, > 3). The significance level of the statistical tests was set at 5%.

RESULTS

Year-round environmental conditions

Environmental conditions varied markedly with salinity along the estuary throughout the year. Turbidity usually exceeded 200 NTU at salinities 0.1–10 with highest values (up to 1000 NTU) at salinity 0.1–3 (Fig. 2A, Table I). The scatter pattern of POC concentrations differed from that of chlorophyll *a*, but corresponded closely with that of phaeopigments (Fig. 2B–D). Concentrations of POC and phaeopigments were usually higher at salinities 0.1–3 under high turbidity conditions (Table I). Significant differences among salinity ranges combined with no clear seasonal change were found in turbidity, POC and phaeopigments (two-way ANOVA, $P < 0.05$, Table II). On the contrary, higher chlorophyll *a* concentrations were found at salinities < 0.1 and > 20 under low turbidity conditions (Table I). Chlorophyll *a* reached high concentrations mainly in the warm season (Table I). However, a significant interaction between salinity and temperature (two-way ANOVA, $P < 0.05$, Table II) obscured their respective effects on chlorophyll *a* concentrations.

Low POC:chlorophyll *a* ratios (< 100) occurred only at salinities < 0.1 and > 20 in the warm season, indicating the dominance of phytoplankton in POC (Fig. 2E). The $\delta^{13}C$ values in POC were depleted (< -25‰) at salinities < 0.1 and enriched (> -23‰) at salinities > 20 in the warm season (Fig. 2F). This was in contrast with relatively constant $\delta^{13}C$ values (-25–-23‰) in POC at salinities 0.1–20 throughout the year (Table I). However, effects of salinity and temperature were not statistically confirmed in POC:chlorophyll *a* ratios and POC $\delta^{13}C$ values because of significant interactions between salinity and temperature (two-way ANOVA, $P < 0.05$, Table II). Exceptionally low salinities were observed through the tidal reach under recurring flood conditions in July 2006

(cf. Suzuki *et al.*, 2012b). Lowest concentrations of POC, chlorophyll *a* and phaeopigments were accompanied by high POC:chlorophyll *a* ratios (> 1000) and constant $\delta^{13}C$ values in POC (-24.5‰) in the flood period.

Year-round comparisons between *S. sinensis* and *P. inopinus*

Irrespective of the season, *S. sinensis* exhibited a unimodal pattern of distribution along the estuarine salinity gradient, exceeding 1 ind. L^{-1} at salinities 0.1–10 with highest densities close to salinity 1 (Fig. 3A). The $\delta^{13}C$ values in *S. sinensis* were distributed over -30–-25‰ and slightly more enriched at higher salinities (Spearman's correlation coefficient: $r_s = 0.45$, $P < 0.05$). The majority of C:N ratios in *S. sinensis* ranged from 4.5 to 5.5, although higher C:N ratios occurred at higher salinities ($r_s = 0.38$, $P < 0.05$). In contrast to *S. sinensis*, *P. inopinus* exhibited a clear seasonal change in density, exceeding 1 ind. L^{-1} almost only in the warm season (> 20ºC; Fig. 3B). Such high densities of *P. inopinus* were observed over a relatively wide salinity range (0.1–20). The $\delta^{13}C$ values in *P. inopinus* were strongly correlated with salinity ($r_s = 0.71$, $P < 0.05$): -30–-27‰ at lower salinities and -26–-23‰ at higher salinities. The C:N ratios in *P. inopinus* ranged from 4.5 to 5.5 without a clear relationship with salinity ($r_s = -0.08$, $P > 0.05$). No seasonal change was evident in $\delta^{13}C$ values or C:N ratios in both *S. sinensis* and *P. inopinus*.

In *S. sinensis*, $\Delta\delta^{13}C$ values were scattered over -7–-1‰ and weakly correlated with salinity ($r_s = 0.29$, $P < 0.05$; Fig. 4A). Lower $\Delta\delta^{13}C$ values, by definition, represent larger differences in $\delta^{13}C$ between copepods and POC. Although $\Delta\delta^{13}C$ values were significantly lower at higher phaeopigment concentrations ($r_s = -0.33$, $P < 0.05$), they were not correlated with any other environmental parameters considered in *S. sinensis* (Table III). *Pseudodiaptomus inopinus* held significantly lower $\Delta\delta^{13}C$ values (-7–-1‰) at salinities 0.1–3 compared with higher $\Delta\delta^{13}C$ values (-4–1‰) outside this salinity range (Kruskal-Wallis test, $P < 0.05$; Fig. 4B). The $\Delta\delta^{13}C$ differences among the salinity ranges were less obvious in the cold season (< 20ºC) when *P. inopinus* seldom occurred at salinities < 0.1. In contrast with *P. inopinus*, such $\Delta\delta^{13}C$ differences among the salinity

ranges were not observed in *S. sinensis* (Kruskal-Wallis test, $P > 0.05$). Whereas *P. inopinus* held significantly lower $\Delta\delta^{13}C$ values at higher turbidities and higher concentrations of POC and phaeopigments throughout the year (r_s = -0.28, -0.61 and -0.60, respectively; $P < 0.05$; Table III), this species held higher and less variable $\Delta\delta^{13}C$ values at lower POC:chlorophyll *a* ratios in the warm season (> 20ºC).

Responses to short-term environmental changes

In summer 2005, the daily freshwater discharge was small (< 50 m^3 s^{-1}) in June before causing a large flood event (up to 1768 m^3 s^{-1}) in early July (Fig. 5A). The discharge gradually settled down to the previous level by late July. Before the flood, chlorophyll *a* concentrations were highest close to the upper limit of the tidal reach in contrast with high phaeopigment concentrations observed slightly more downstream (Fig. 5B). Both chlorophyll *a* and phaeopigment concentrations decreased drastically during the flood, whereas after the flood they regained similar spatial patterns to those observed before the flood. The $\delta^{13}C$ values in POC remained relatively enriched (> -26‰) at all stations before and during the flood, with the exception of the most upstream station on 24 June (Fig. 5C). After the flood, more depleted $\delta^{13}C$ values in POC (< -26‰) occurred especially at upstream stations. The $\delta^{13}C$ values in both *S. sinensis* and *P. inopinus* apparently reflected the spatiotemporal variability in POC $\delta^{13}C$: relatively enriched at all stations before the flood and relatively depleted at upstream stations after the flood.

Different responses to flood-induced environmental changes between *S. sinensis* and *P. inopinus* were revealed when the data were analyzed in relation to salinity. Except during the flood, high chlorophyll *a* concentrations (> 20 μg L^{-1}), combined with low POC:chlorophyll *a* ratios (< 100), were observed close to salinity 0.1, in contrast with high phaeopigment concentrations (> 20 μg L^{-1}) observed at salinities 0.1–3 (Fig. 6A–C). The $\delta^{13}C$ values in POC were relatively enriched (> -26‰) over the whole salinity range before the flood, whereas after the flood they were drastically depleted (< -26‰) at salinities < 0.1 (Fig. 6D). During the flood, the whole sampling

area was dominated by fresh water characterized by low concentrations of chlorophyll *a* and phaeopigments, high POC:chlorophyll *a* ratios and constant POC $\delta^{13}C$ values (ca. -24‰). Irrespective of salinity, *S. sinensis* showed completely different $\delta^{13}C$ values before and after the flood (> -27‰ and < -27‰, respectively; Fig. 6E). Although *P. inopinus* also showed relatively enriched $\delta^{13}C$ values (> -27‰) over the whole salinity range before the flood, this species exhibited a strong correlation of $\delta^{13}C$ with salinity ($r_s = 0.84$, $P < 0.05$) after the flood (Fig. 6F). This strong correlation was primarily attributable to $\delta^{13}C$ values in *P. inopinus* observed outside the salinity range of 0.1–3.

DISCUSSION

Food sources for the sympatric oligohaline copepods *S. sinensis* and *P. inopinus* were estimated from detailed comparisons of spatiotemporal variability in $\delta^{13}C$ between copepods and POC. Both copepods selectively utilize freshwater/oligohaline phytoplankton and/or its detritus in the ETM, whereas only *P. inopinus* utilizes meso/polyhaline phytoplankton downstream from the ETM. To estimate food sources accurately, preconditions for the interpretation of $\delta^{13}C$ data are discussed first. Dependence on differential food sources is discussed between the two copepods in light of their respective patterns of spatial occurrence relative to the ETM.

Preconditions for interpretation

Spatial heterogeneity in environmental conditions along the salinity gradient of the Chikugo River estuary was clearly demonstrated in the present study as compared with previous studies (Suzuki *et al.*, 2007, 2009, 2012b). High turbidities, accompanied by high concentrations of POC and phaeopigments, always occurred at salinities 0.1–3. Therefore we define the ETM by the salinity range of 0.1–3. Up- and downstream from the ETM, the dominance of living phytoplankton was often indicated by high chlorophyll *a* concentrations and low POC:chlorophyll *a* ratios especially in the warm season. Phytoplankton production is probably facilitated in relatively transparent and

298 warm waters (Suzuki *et al.*, 2012b; Yokoyama *et al.*, 2012). On the contrary, terrestrial-plant and
299 phytoplankton detritus is likely to accumulate in the ETM throughout the year (Suzuki *et al.*,
300 2012b). Generally, such spatiotemporal changes in environmental conditions are considered
301 common in macrotidal estuaries where the ETM develops markedly (Irigoien and Castel, 1997;
302 Lemaire *et al.*, 2000; Modéran *et al.*, 2010; Savoye *et al.*, 2012).

303 To interpret spatiotemporal variability in stable isotope ratios in animals, it is important to
304 determine both fractionation and turnover of stable isotope ratios in animals through laboratory
305 experiments (Gannes *et al.*, 1997). Since such experiments were not conducted in the present study,
306 the fractionation and turnover of $\delta^{13}C$ in copepods are estimated from our field observations and the
307 literature. The fractionation of $\delta^{13}C$ is generally small between animals and their diet (i.e. ±1‰;
308 DeNiro and Epstein, 1978; Fry and Sherr, 1984), whereas it is often affected by the lipid content of
309 animals due to distinctly depleted $\delta^{13}C$ values in lipids (DeNiro and Epstein, 1977). Differences in
310 lipid content can be responsible for variability in $\delta^{13}C$ within a copepod species (Matthews and
311 Mazumder, 2005; Smyntek *et al.*, 2007). However, relatively low and invariable C:N ratios indicate
312 that lipid content is unlikely to have affected copepod $\delta^{13}C$ values in the present study. Therefore it
313 is possible to estimate the fractionation of $\delta^{13}C$ between copepods and their food sources at ±1‰
314 (DeNiro and Epstein, 1978; Fry and Sherr, 1984). Although food quality can be another
315 complicating factor in isotopic fractionation, laboratory experiments are necessary to obtain the
316 relevant information (cf. Aberle and Malzahn, 2007).

317 In response to flood-induced environmental changes in summer 2005, copepod $\delta^{13}C$ values
318 varied drastically between late June and late July (or early August), although new cohorts could
319 have replaced old ones to some extent (Suzuki *et al.*, 2012a). This indicates that copepod $\delta^{13}C$
320 values possibly reflect environmental changes within several weeks in the warm season. Based on
321 high growth rates reported in *Sinocalanus tenellus* (Kimoto *et al.*, 1986) and *Pseudodiaptomus*
322 *marinus* (Uye *et al.*, 1983) at 20ºC, the $\delta^{13}C$ values in *S. sinensis* and *P. inopinus* should converge
323 on a $\delta^{13}C$ value in new diet within one week after a diet switch (cf. Klein Breteler *et al.*, 2002).

Such quick turnover rates of $\delta^{13}C$ in copepods would justify our simple method in which copepod $\delta^{13}C$ values were compared with POC $\delta^{13}C$ values without taking account of time lag. At lower temperatures, turnover rates of $\delta^{13}C$ in copepods may be slower due to lower growth and metabolic rates (cf. Tamelander *et al.*, 2006). Nevertheless, our overlooking of time lag is unlikely to have affected the results considerably, because POC $\delta^{13}C$ values were less variable in the cold season. Our monthly comparisons of copepod $\delta^{13}C$ with POC $\delta^{13}C$ would therefore provide reliable information about food sources for copepods throughout the year.

Food sources for *S. sinensis* and *P. inopinus*

In the ETM, *S. sinensis* and *P. inopinus* are considered to prefer freshwater/oligohaline phytoplankton and its detritus over terrestrial-plant detritus. The $\delta^{13}C$ values in terrestrial-plant detritus are relatively constant (ca. -24‰), as is well represented in POC $\delta^{13}C$ values during floods (Suzuki *et al.*, 2012b). Consequently, more depleted $\delta^{13}C$ values observed in copepods throughout the year should be attributable to freshwater/oligohaline phytoplankton. This idea is supported by temporal changes in copepod $\delta^{13}C$ values observed during the intensive sampling campaign. As the $\delta^{13}C$ values in freshwater/oligohaline phytoplankton became depleted after the flood, *S. sinensis* and *P. inopinus* gradually displayed depleted $\delta^{13}C$ values in the ETM, probably feeding on freshwater/oligohaline phytoplankton and its detritus. Generally, feeding preferences of copepods for phytoplankton and its detritus are considered common in many estuaries (Tackx *et al.*, 2003; Martineau *et al.*, 2004; Hoffman *et al.*, 2008). Copepods feed selectively on more nutritious food sources (Cowles *et al.*, 1988; DeMott, 1995; Tackx *et al.*, 1995), although selection processes may differ among species (Richman *et al.*, 1977; Irigoien *et al.*, 1996). Feeding on terrestrial-plant detritus is nevertheless attractive especially in the cold season when phytoplankton is scarce (Suzuki *et al.*, 2012b). Specifically, *S. sinensis* might utilize terrestrial-plant detritus to maintain its year-round large biomass in the ETM (Suzuki *et al.*, 2012a, 2013).

The stenohaline copepod *S. sinensis* always concentrated close to salinity 1 in the ETM,

where terrestrial-plant and phytoplankton detritus accumulated throughout the year (Suzuki *et al.*, 2012b). The $\Delta\delta^{13}C$ values in *S. sinensis* were usually distant from the estimated fractionation of $\delta^{13}C$ between copepods and their food source (i.e. ±1‰). Given that larger differences in $\delta^{13}C$ between copepods and POC are associated with greater selectivity in feeding and/or assimilation by copepods (cf. Del Giorgio and France, 1996), *S. sinensis* is considered to be highly selective about its food source in favor of freshwater/oligohaline phytoplankton and its detritus. Moreover, the total absence of relatively enriched $\delta^{13}C$ values (> -24‰) in *S. sinensis* indicates little or no dependence on meso/polyhaline phytoplankton. We argue that the feeding strategy of *S. sinensis* is closely linked to the accumulation of detritus in the ETM, which constitutes an irreplaceable habitat for *S. sinensis* compared with other estuarine environments (Suzuki *et al.*, 2012a, 2013). In contrast, the less stenohaline copepod *P. inopinus* occurred not only in the ETM but also in its surrounding waters. Up- and downstream from the ETM, this species often exhibited $\Delta\delta^{13}C$ values of ±1‰, clearly reflecting $\delta^{13}C$ values in phytoplankton. The most enriched $\delta^{13}C$ values in *P. inopinus* were comparable with the average $\delta^{13}C$ value in the herbivorous copepod *Acartia omorii* in the lower estuary (Uchima, 1988; Suzuki *et al.*, 2008). These findings suggest that *P. inopinus* feeds unselectively on living phytoplankton outside the ETM whereas in the ETM *P. inopinus* is selective about its food source like *S. sinensis*.

CONCLUSIONS

The present study demonstrates that freshwater/oligohaline phytoplankton and its detritus constitute the main food sources for both *S. sinensis* and *P. inopinus* in the ETM, although the possible contribution of terrestrial-plant detritus to their production remains to be determined. The less stenohaline copepod *P. inopinus* utilizes relatively diverse food sources depending on environmental conditions along the estuarine salinity gradient. Feeding on living phytoplankton outside the ETM, combined with an egg-carrying strategy in reproduction, could explain the large biomass of *P. inopinus* during the warm season (Suzuki *et al.*, 2012a). In contrast, the stenohaline

copepod *S. sinensis* is restricted within the ETM and unlikely to utilize meso/polyhaline phytoplankton. To estimate the relative contribution of phytoplankton and terrestrial plants to copepod production, additional tracers will be useful (e.g. fatty acid composition, ^{13}C and ^{15}N labeling). It is also a challenge to reveal biological mechanisms of *S. sinensis* underlying its year-round large biomass in the ETM (e.g. ecological and physiological tolerance for high turbidities). As the production of larval and juvenile fish is entirely supported by *S. sinensis* and *P. inopinus* in the Chikugo River estuary (Hibino *et al.*, 1999; Suzuki *et al.*, 2008, submitted), further investigations on the two copepods will improve our understanding of the productive food web in the ETM.

FUNDING

The present study was partially supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Mr S. Koga of Okinohata Fisheries Cooperative Association and Mr T. Tsukamoto of Shimochikugo Fisheries Cooperative Association for their assistance with field sampling. We are also grateful to Fukuoka Fisheries & Marine Technology Research Center. Dr A. Kasai of the Laboratory of Fisheries and Environmental Oceanography, Kyoto University, provided us with technical advice concerning stable carbon isotope ratios. Dr J. Shoji of Takehara Fisheries Research Laboratory, Hiroshima University, Dr T. Wada of Fukushima Prefectural Fisheries Experimental Station, Dr R. Sugimoto of the Research Center for Marine Bioresources, Fukui Prefectural University, and former students in our laboratory helped with field sampling. Mr. J. Grigor kindly reviewed the manuscript in terms of English grammar. Photosynthetic pigments and stable carbon isotope ratios were analyzed at the Laboratory of Marine Environmental Microbiology and the Center for Ecological Research, Kyoto University, respectively.

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550

551 **LEGENDS**

552 **Table I:** *Average of turbidity, particulate organic carbon (POC), chlorophyll a (Chl. a),*
553 *phaeopigments (Phaeo.), POC:Chl. a ratios, and stable carbon isotope ratios ($\delta^{13}C$) observed in*
554 *the Chikugo River estuary from March 2005 to December 2006. The average values were*
555 *calculated for each combination of salinity (S <0.1, 0.1–3, 3–20, >20) and temperature (T <20ºC,*
556 *>20ºC) ranges.*

557

558 **Table II:** *Summary of the two-way repeated-measures analysis of variance of environmental*
559 *parameters observed in the Chikugo River estuary from March 2005 to December 2006. Four*
560 *salinity ranges (S <0.1, 0.1–3, 3–20, >20) and two temperature ranges (T <20ºC, >20ºC) were*
561 *set to assess the spatiotemporal variability of the environmental parameters.*

562

563 **Table III:** *Spearman's correlation coefficients of $\Delta\delta^{13}C$ in S. sinensis and P. inopinus relative to*
564 *salinity, turbidity, particulate organic carbon (POC), chlorophyll a (Chl. a), phaeopigments*
565 *(Pheo.) and POC:Chl. a ratios in the Chikugo River estuary from March 2005 to December 2006.*
566 *$\Delta\delta^{13}C$ values were calculated by subtracting $\delta^{13}C$ in POC from $\delta^{13}C$ in copepods at each station*
567 *on each sampling date. Significant coefficients are indicated by asterisks.*

568

569 **Fig. 1.** Sampling stations along the Chikugo River estuary on Kyushu Island in southwestern Japan.
570 Black and white circles represent our regular and extra sampling stations, respectively. The
571 Chikugo Weir is represented by a black rectangle.

572

573 **Fig. 2.** Environmental conditions observed along the salinity gradient of the Chikugo River estuary
574 from March 2005 to December 2006. Turbidity (**A**), particulate organic carbon (POC)
575 concentrations (**B**), chlorophyll *a* concentrations (**C**), phaeopigment concentrations (**D**),
576 POC:chlorophyll *a* ratios (**E**) and stable carbon isotope ratios ($\delta^{13}C$) in POC (**F**) are shown.

577 Crosses and exes represent values observed in the cold (< 20ºC) and warm (> 20ºC) seasons,
578 respectively.
579
580 **Fig. 3.** Adult/copepodid density, stable carbon isotope ratios ($\delta^{13}C$) and carbon to nitrogen atomic
581 ratios (C:N) observed in *S. sinensis* (**A**) and *P. inopinus* (**B**) along the salinity gradient of the
582 Chikugo River estuary from March 2005 to December 2006. Black and white symbols represent
583 values observed in the cold (< 20ºC) and warm (> 20ºC) seasons, respectively. Spearman's
584 correlation coefficients are indicated by r_s.
585
586 **Fig. 4.** Differences in $\delta^{13}C$ ($\Delta\delta^{13}C$) between copepods and particulate organic carbon (POC)
587 observed in *S. sinensis* (**A**) and *P. inopinus* (**B**) in the Chikugo River estuary from March 2005 to
588 December 2006. $\Delta\delta^{13}C$ values are shown in relation to salinity, phaeopigment concentrations and
589 POC:chlorophyll *a* ratios. See Fig. 3 for details.
590
591 **Fig. 5.** Temporal changes in freshwater discharge (**A**), in horizontal distributions of chlorophyll *a*
592 and phaeopigments (**B**), and in interrelations of stable carbon isotope ratios ($\delta^{13}C$) among
593 particulate organic carbon (POC), *S. sinensis* and *P. inopinus* (**C**) observed along the Chikugo
594 River estuary in summer 2005. Arrows indicate sampling dates.
595
596 **Fig. 6.** Environmental conditions and stable carbon isotope ratios ($\delta^{13}C$) observed along the salinity
597 gradient of the Chikugo River estuary in summer 2005. Chlorophyll *a* concentrations (**A**),
598 phaeopigment concentrations (**B**), POC:chlorophyll *a* ratios (**C**), $\delta^{13}C$ in POC (**D**), $\delta^{13}C$ in *S.*
599 *sinensis* (**E**) and $\delta^{13}C$ in *P. inopinus* (**F**) are shown. Black, white and grey symbols represent
600 values observed before, during and after the flood of early July, respectively.
601
602

Table I: *Average of turbidity, particulate organic carbon (POC), chlorophyll a (Chl. a), phaeopigments (Phaeo.), POC:Chl. a ratios, and stable carbon isotope ratios ($\delta^{13}C$) observed in the Chikugo River estuary from March 2005 to December 2006. The average values were calculated for each combination of salinity (S <0.1, 0.1–3, 3–20, >20) and temperature (T <20ºC, >20ºC) ranges.*

	Turbidity (NTU)		POC (mg L^{-1})		Chl. *a* (µg L^{-1})		Phaeo. (µg L^{-1})		POC:Chl. *a*		$\delta^{13}C$ (‰)	
	T<20ºC	T>20ºC	T<20ºC	T>20ºC	T<20ºC	T>20ºC	T<20ºC	T>20ºC	T<20ºC	T>20ºC	T<20ºC	T>20ºC
S<0.1	62	57	1.8	1.4	6.6	13.4	3.7	5.3	357	442	-25.3	-26.1
0.1<S<3	413	461	10.1	7.3	8.8	17.8	22.5	20.0	1726	693	-24.4	-24.0
3<S<20	132	228	3.1	3.2	4.5	7.0	7.4	9.4	1094	845	-24.1	-23.3
S>20	36	101	1.1	1.8	2.3	11.9	2.8	3.4	664	209	-23.3	-22.3

Table II: *Summary of the two-way repeated-measures analysis of variance of environmental parameters observed in the Chikugo River estuary from March 2005 to December 2006. Four salinity ranges (S <0.1, 0.1–3, 3–20, >20) and two temperature ranges (T <20ºC, >20ºC) were set to assess the spatiotemporal variability of the environmental parameters.*

	Turbidity (df_E = 181)	POC (df_E = 210)	Chl. *a* (df_E = 210)	Phaeo. (df_E = 210)	POC:Chl. *a* (df_E = 210)	$\delta^{13}C$ (df_E = 209)
S (df_S = 3)	***F* = 48.7** ***P* < 0.01**	***F* = 70.8** ***P* < 0.01**	***F* = 7.3** ***P* < 0.01**	***F* = 37.0** ***P* < 0.01**	***F* = 20.8** ***P* < 0.01**	***F* = 67.0** ***P* < 0.01**
T (df_T = 1)	*F* = 1.2 *P* = 0.27	*F* = 0.2 *P* = 0.62	***F* = 40.3** ***P* < 0.01**	*F* = 0.0 *P* = 0.89	***F* = 38.4** ***P* < 0.01**	***F* = 5.4** ***P* = 0.02**
S×T ($df_{S×T}$ = 3)	*F* = 1.9 *P* = 0.13	*F* = 2.2 *P* = 0.09	***F* = 7.1** ***P* < 0.01**	*F* = 1.8 *P* = 0.15	***F* = 2.7** ***P* = 0.05**	***F* = 6.7** ***P* < 0.01**

The *F* and *P* values were calculated after logarithmic transformation for turbidity, particulate organic carbon (POC), chlorophyll *a* (Chl. *a*), phaeopigments (Phaeo.), POC:Chl. *a* ratios and stable carbon isotope ratios ($\delta^{13}C$). Degrees of freedom for salinity (df_S), temperature (df_T), interaction between them ($df_{S×T}$) and error (df_E) are given in parentheses. Boldface type indicates statistically significant values.

Table III: *Spearman's correlation coefficients of $\Delta\delta^{13}C$ in S. sinensis and P. inopinus relative to salinity, turbidity, particulate organic carbon (POC), chlorophyll a (Chl. a), phaeopigments (Pheo.) and POC:Chl. a ratios in the Chikugo River estuary from March 2005 to December 2006. $\Delta\delta^{13}C$ values were calculated by subtracting $\delta^{13}C$ in POC from $\delta^{13}C$ in copepods at each station on each sampling date. Significant coefficients are indicated by asterisks.*

	Salinity	Turbidity	POC	Chl. *a*	Pheo.	POC:Chl. *a*
$\Delta\delta^{13}C$ in *S. sinensis*	0.29*	-0.02	-0.24	-0.02	-0.33*	-0.12
$\Delta\delta^{13}C$ in *P. inopinus*	0.27	-0.28*	-0.61*	-0.09	-0.60*	-0.35*

Fig. 1

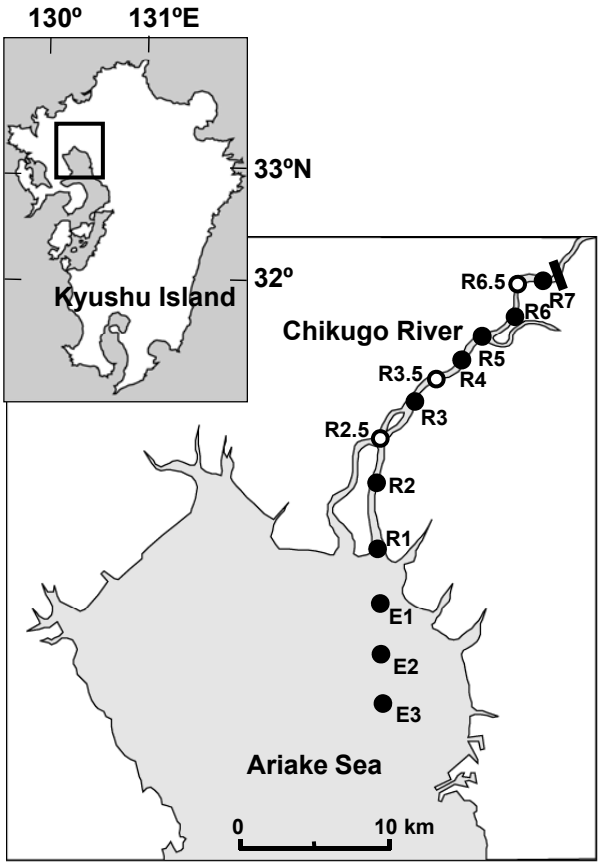

Fig. 2

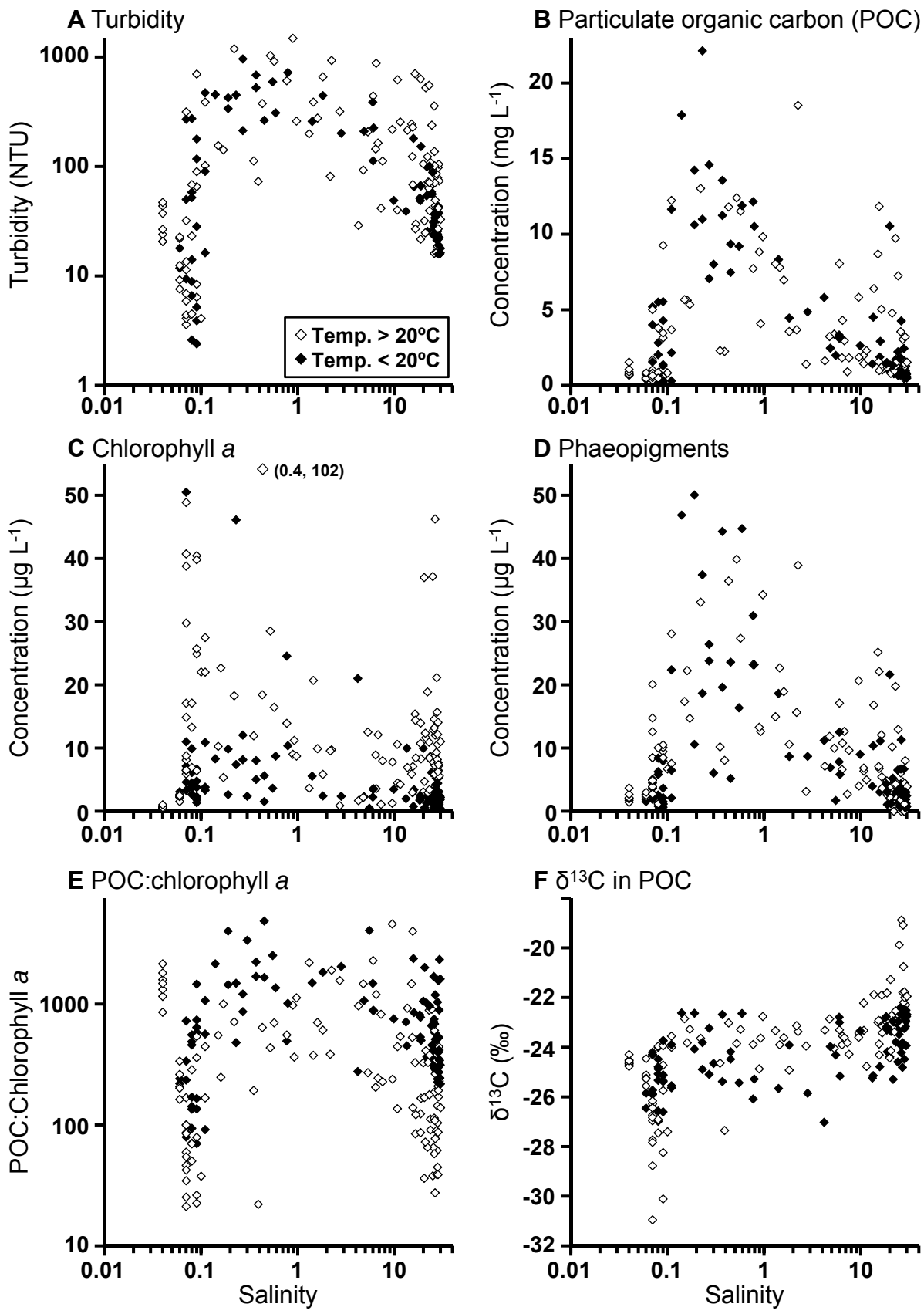

Fig. 3

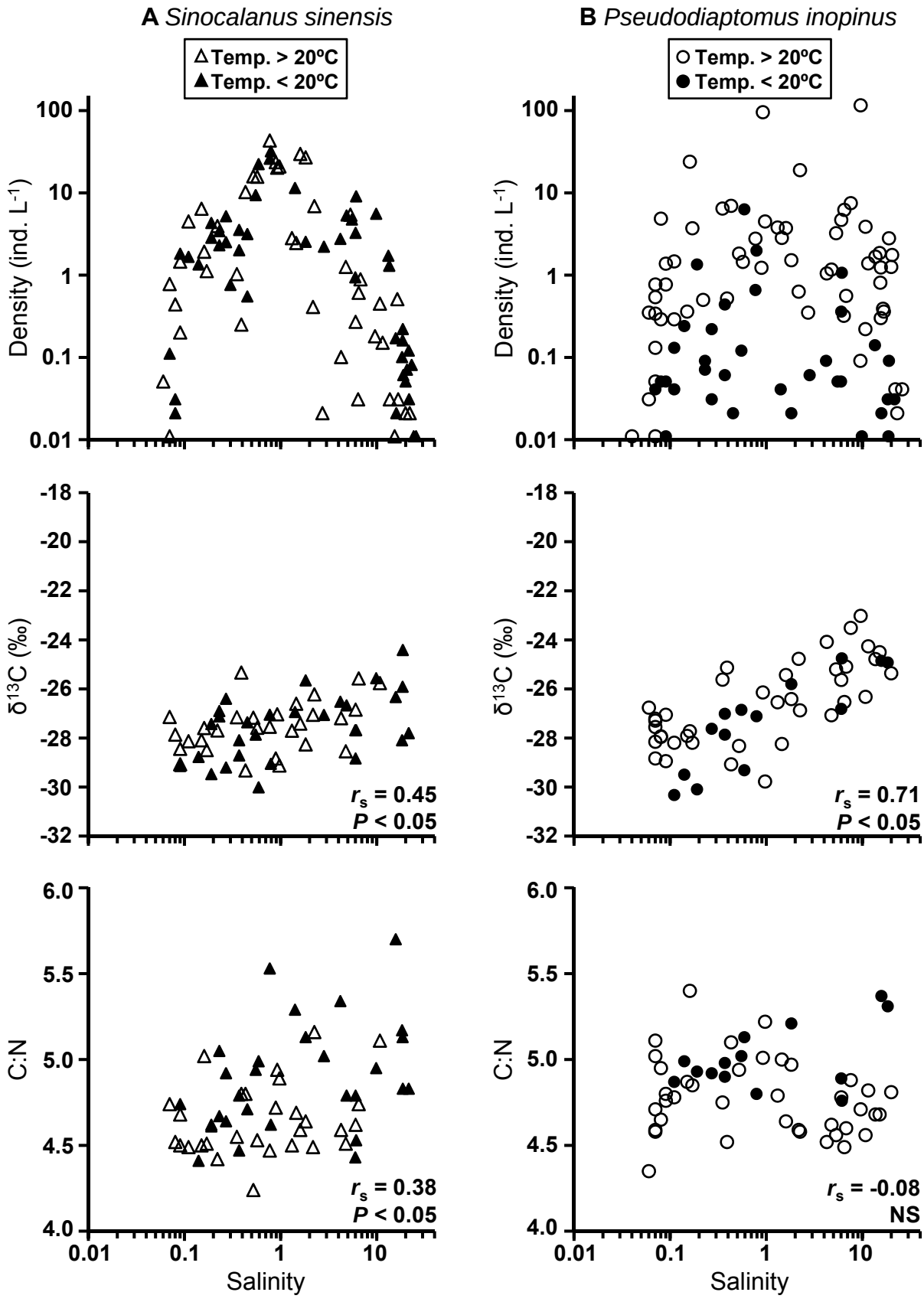

Fig. 4

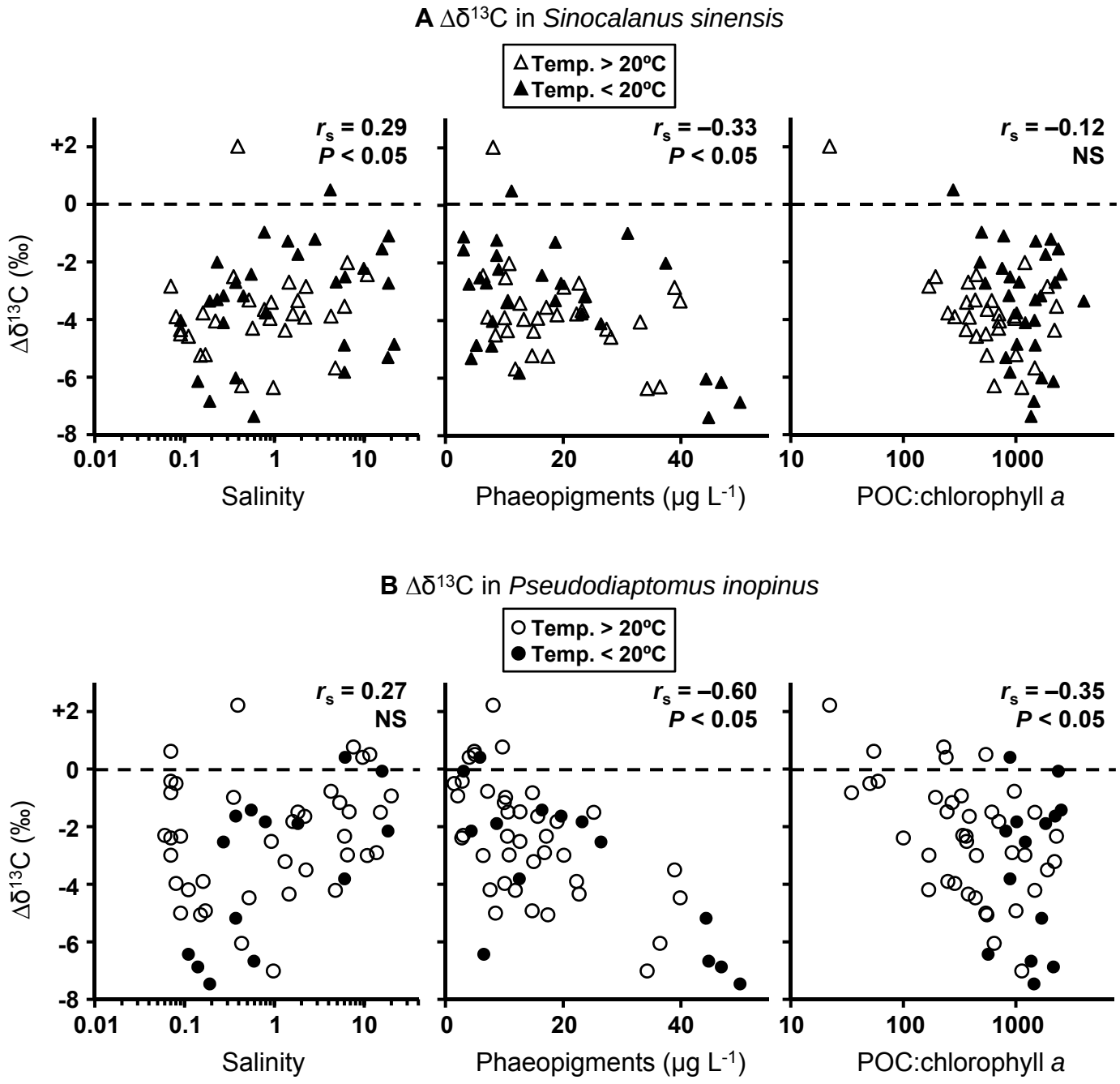

Fig. 5

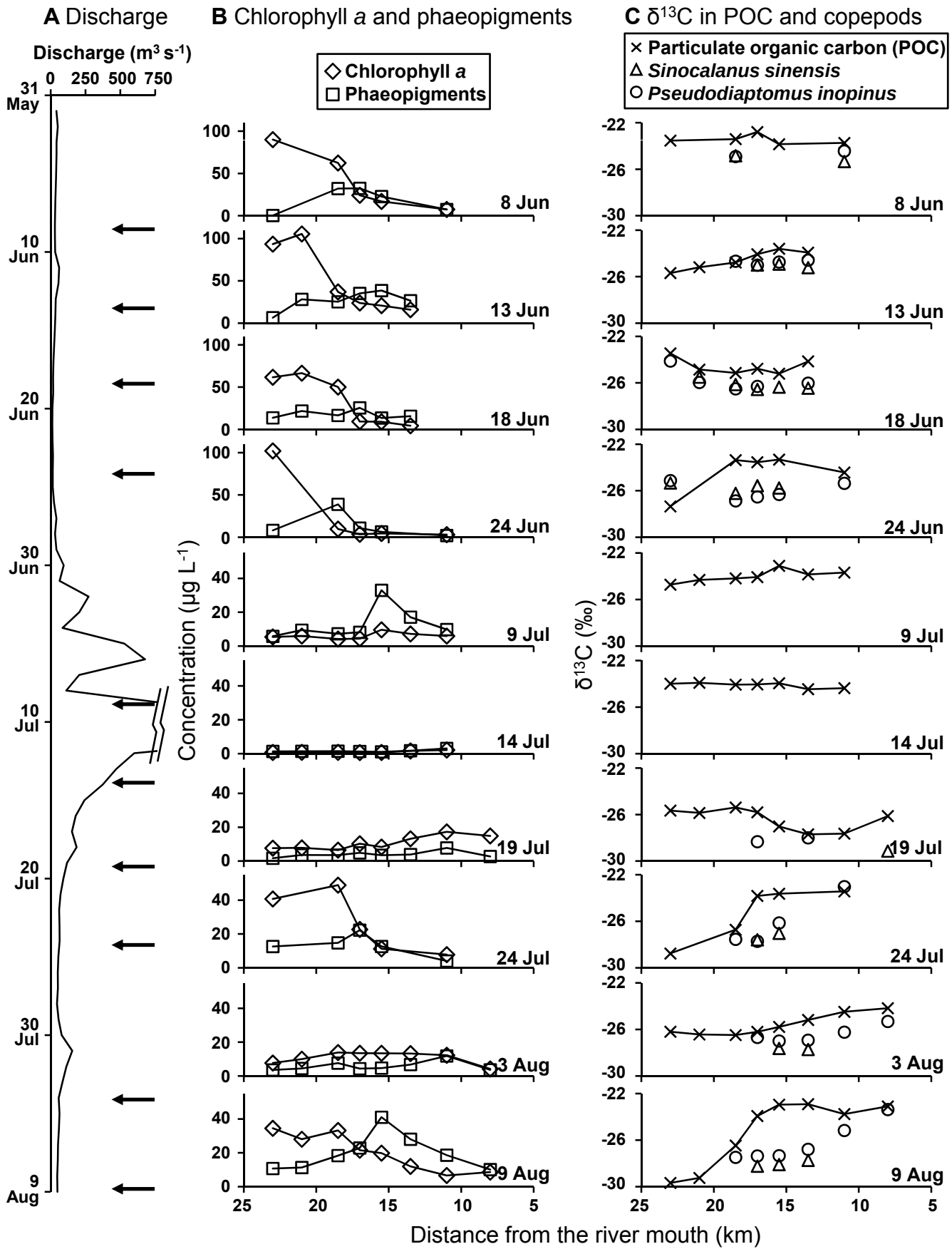

Fig. 6